Advancing Space Flight Medical Care Through On-demand Protein Therapeutic Production Capabilities

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Abstract –

Cell-free protein synthesis (CFPS) is an in vitro protein synthesis system that provides a uniquely adaptable platform for protein production. In recent years, lyophilization of CFPS have expanded the capabilities of this system by improving reagent stability outside of cold-chain storage. This project sought to utilize lyophilized CFPS reagents to develop a convenient, shelf-stable, on-demand therapeutic protein production platform. The therapeutic protein production potential of this novel platform was demonstrated by the successful expression of an FDA approved therapeutic protein. With the advancement of shelf-stable, on-demand protein therapeutic production platforms comes the potential to provide point-of-care medical treatment for space-flight crews during long duration missions.

Introduction

As the dream of long-term space exploration becomes a reality, so do the unique health risks associated with space flight. Missions requiring long-duration habitation beyond low-earth orbit pose significant challenges to the health and well-being of the crew. Successful medical interventions, as well as biomedical countermeasures to prevent or mitigate high-risk conditions, are contingent on the therapeutics available to the crew. Some of the most beneficial options for medical care are protein therapeutics, as they provide particularly effective treatments for cancer, genetic diseases, neurological disorders, and radiation syndromes. However, the availability of protein therapeutics is limited by their low stability under high-levels of radiation and long term storage [1].

This project sought to overcome the challenges of protein therapeutic availability during long-term space flight by engineering a lyophilized cell-free protein synthesis (CFPS) system capable of producing protein therapeutics on demand and with minimal equipment; thus, providing life-saving treatments to space flight crews.

Relevance to NASA Aims

This work focuses on supporting Strategic Goal 2 of NASA's 2018 Strategic Plan which is to "Extend Human Presence Deeper into Space and to the Moon for Sustainable Long-term Exploration and Utilization" [2]. In particular, this project addresses the specific challenges identified in the NASA Technology Roadmaps: pharmaceutical stability under space flight conditions (TA 6.3.2), and effective treatment options for high-risk medical conditions for long-duration health (TA 6.5.2) [3]. Potentially, many of these challenges can be addressed through on-demand production of protein therapeutics during long-term space flight.

Protein therapeutic availability on board spacecraft can improve treatment options and countermeasures during interplanetary missions against cancer, genetic diseases, neurological disorders, and radiation syndromes (TA 6.5.2.4, 6.5.2.2, 6.5.2.1). However, the availability of these powerful therapeutics during missions is

limited by their long-term stability under space-flight conditions and the difficulty of determining the therapeutics one will need prior to take-off. On-demand protein therapeutic production could eliminate the need to store protein therapeutics long-term while also improving the versatility of in-flight biomedical countermeasures and treatments

Background

Cell-free Protein Synthesis

The production of proteins requires biological processes as conducted by specialized cellular machinery. The traditional method of protein manufacturing produces proteins within living cell cultures, or *in vivo*. These cell-based production platforms are dependent on the growth and replication of living cells and therefore require time intensive production, purification, and protein isolation steps. Protein therapeutics are conventionally purified and stored in aqueous or dried form in low temperature storage until administration. However, stability of these isolated proteins is limited over time with elevated temperatures. *In vitro* cell-free protein synthesis systems (CFPS) are an emerging production platform that provide an alternative approach to conventional protein production and storage. CFPS platforms circumvent many of the disadvantages of *in vivo* manufacturing and present the capability of on-demand protein therapeutic production.

On-demand protein therapeutic production through CFPS may enable spaceflight crews to produce protein therapeutics as the need arises as opposed to relying on spacecraft inventory which may be limited by payload restrictions and therapeutic stability limitations. Protein therapeutics are most commonly expressed in vivo; however, cellular systems are not particularly effective in the space environment due to cell destruction under ionizing radiation [4]. These in vivo systems also require a significant amount of equipment and complex purification steps. Alternatively, CFPS systems make it possible to synthesize proteins outside of a cell by combining the DNA of the protein of interest with emancipated cellular machinery in a reaction vessel (Figure 1). Cell-free expression has been demonstrated on a wide array of therapeutic proteins including antibodies, vaccines, and cancer drugs [5]. CFPS technology facilitates rapid product purification with minimal equipment and provides an efficient protein production platform with high protein yields in as little as an hour which is an order of magnitude faster than in vivo production [6].

Lyophilization

CFPS provides rapid recombinant protein production; however, the major components of a cell free system must be stored in aqueous solutions below freezing [7]. To ensure protein synthesis viability, the CFPS solutions are most

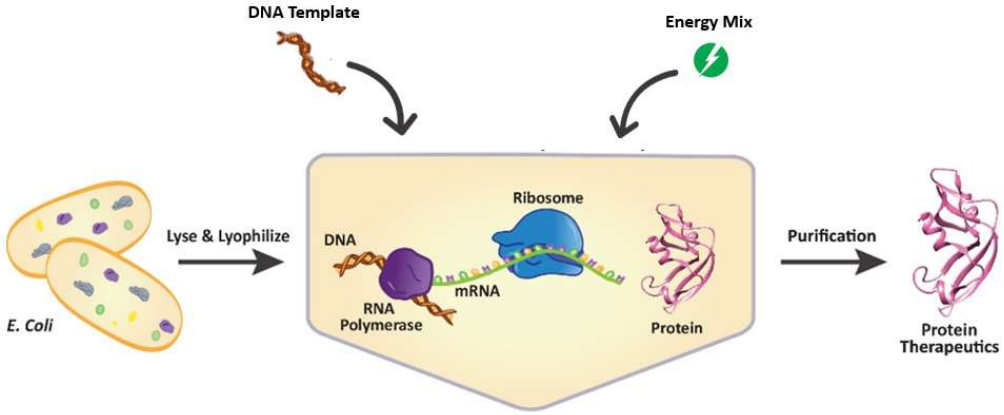

Figure 1: Illustration of CFPS: E. Coli cells are lysed for components to be used in a CFPS system for the production of protein therapeutics or other biocatalysts.

commonly maintained at -80°C, requiring low temperature freezers. To overcome this drawback, lyophilization can be used to preserve CFPS systems. Lyophilization, or freeze-drying, is a commonly used preservation technique on consumables to extend shelf-life, reduce volume and weight, and lower bacterial contamination. We can utilize this technique on CFPS systems with minimal disruption in efficacy as they do not require living cells. A CFPS system can therefore be created from lyophilized, shelf-stable reagents (Figure 4) [8]. These ready-made powdered CFPS systems are capable of high-density, thermostable storage with the proven capacity to produce protein therapeutics [9]. An example of a stored, small-scale lyophilized CFPS system is shown in Figure 2.

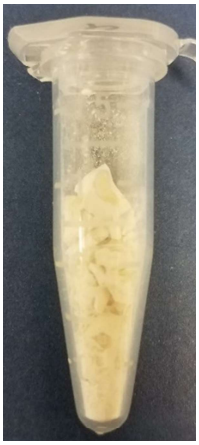

Figure 2: Image of Lyophilized CFPS system stored in test tube 6 months at room temperature

Initial lyophilization work using freeze-dried cell extracts and separate powdered energy systems demonstrated that the lyophilized systems retain viability over a 60-day period at room temperatures [8] and in follow on work has demonstrated retained activity after 1 year when stored at 4°C. In comparison, all activity was lost in the non-lyophilized sample [9]. Additionally, cryoprotectant additives can be considered for even greater retention of CFPS activity over longer storage periods. Initial studies using cryoprotectant have shown promise for prolonged storage [10].

Previous iterations of lyophilized CFPS have required the cell extract and energy mix to be stored separately. One reason for this may be the hydrophobic nature of some of the components of the energy mix which may decrease its dry storage capacity. However, separate storage of the cell extract and energy mix limits the convenience of the current lyophilized CFPS systems. A one-pot CFPS platform which only requires rehydration of water and addition of DNA template would provide a straightforward, user-friendly platform. To this aim, this project sought to optimize lyophilization and cryoprotectant additives to enhance preservation of a one-pot CFPS system.

Convenient Downstream Purification

One of the most significant challenges to on-site, point-of-care production is purification efficiency and the risk of endotoxin exposure from incomplete purification of bacteria residue. Commercial protein therapeutic production relies on extensive purification and quality control steps to ensure complete endotoxin removal. These steps require a high degree of specialized equipment and personnel training. However, an on-demand, point-of-care production platform should be able to produce and purify a protein therapeutic with minimal equipment and training. To address this challenge, our lab recently developed an “endotoxin-free” CFPS system using a commercial LPS-free cell strain ClearColi™BL21(DE3) [9]. By eliminating the source of toxic LPS the risk of endotoxin exposure during therapeutic administration is essentially eliminated.

We have demonstrated the use of ClearColi™ in conventional CFPS systems [11] as well as our lyophilized on-demand CFPS system [12]; however, the preparation of ClearColi™ extract required in the CFPS systems remains time-consuming and labor-intensive because of the relatively slow growth kinetics of the ClearColi™ cell strain. This project sought to develop an improved protocol for the preparation of ClearColi™ cell extract for CFPS systems.

Here we report a streamlined procedure for preparing ClearColi™ cell extract using an auto-induction media (AIM). AIM is an engineered cell culture media which achieves protein expression by catabolite repression of the lacUV5 promoter and eliminates the need for manual induction of protein expression. Culturing Clearcoli™ cells in autoinduction media significantly reduces the hands-on time required during extract preparation and has demonstrated improved extract efficacy.

Experimental Methods

CFPS Reactions

CFPS reactions were formulated with 25 vol percent cell extract, 25 vol percent PANOxSP energy mix as previously described [13], 12 nM plasmid DNA, and ultrapure water to volume. Reaction was incubated 3 h at 37 °C and 120 rpm. Production yields of super-folder green fluorescent protein (sfGFP) were determined by fluorescence at 485/510 excitation/emission wavelengths according to a standard curve created by C14-leucine labeled proteins, as reported previously [7]. Crisantaspase was expressed for 6.5–8 h at 30 °C and 120 RPM and yield was determined using C14 labeled leucine as previously described [7].

Cell Extract Preparation and Lyophilization

Cell-free extracts were prepared from ClearColi™ BL21 (DE3) strains as described previously [2]. Briefly, cells were grown in 2.5 L Tunair flasks (IBI Scientific, Peosta, IA) and production of T7 RNA polymerase was induced via addition of 1mM IPTG during early exponential growth. Cells were harvested in mid log phase by centrifugation at 6000 rcf for 10 min. Cells were homogenized at 21,000 psig in an Avestin French Press. Prior to lyophilization, cryoprotectants were added to cell extract samples as specified in Table 1 [12]. Additionally, the energy system PANOxSP[13] was added to the samples prior to lyophilization.

Table 1
Cryoprotectant content and concentrations [12]

Sample Name	Mass Fraction					
	Glass-former Trehalose	Dextran	Ficoll	Linker Maltitol	Plasticizer DMSO	Total
Control	–	–	–	–	–	0
Trehalose	0.20	–	–	–	–	0.20
Trehalose/DMSO	0.19	–	–	–	0.01	0.20
Dextran (D)	0.20	–	–	–	–	0.20
Dextran/Maltitol/ DMSO (DMD)	–	0.18	–	0.01	0.01	0.20
Ficoll/Maltitol/ DMSO (FMD)	–	–	0.088	0.011	0.011	0.11

Samples were frozen in a -40°C ethanol bath (Just-A-Tilt Shell Freezer Chiller SF-4Az, FTS Systems, Warminster, PA). Samples were then lyophilized (Flexidry MP, FTS Systems). After approximately 1.5 hours of freeze-drying, samples reached minimum weight suggesting samples were sufficiently dried.

Lyophilized CFPS systems were stored at 4°C, 25°C, 37°C, and 50°C for 90 days and tested periodically for protein synthesis activity. Activity was determined by measuring production yields of sfGFP. To demonstrate the capacity of this system to produce protein therapeutics, the FDA approved cancer therapeutic crisantaspase was also produced after 46 days of storage.

Streamlined Endotoxin-free CFPS Preparation

AIM induction kinetics were investigated to determine optimal cell culture fermentation. Auto-induction media was adapted from the "ZYM-5052" protocol reported by Studier [29]. 1 mL overnight culture of BL21 Star™(DE3), and BL21 Star™(DE3)-pY71-sfGFP were was added to 100 mL AIM with either 0.05 or 1% w/v final glucose concentrations. Cultures were fermented at 37°C and 280 rpm. Absorbance (OD_{600}) of cultures were read 2 or 3 times per hour for 12 h. Simultaneously, fluorescence of 100 µl of cell culture were measured with Synergy-MX Multi-Plate Reader (Biotek, Winooski, VT). ClearColi™ BL21(DE3) growth and induction kinetics were assessed similarly

except OD_{600} and culture fluorescence were measured once per hour for 15 h.

After AIM induction kinetics were elucidated, the efficacy of cell extract produced by AIM was tested. ClearColi™ was fermented in 1.5 L AIM prepared to 0.05% final glucose concentration in 2.5 L Tunair shake flasks. Cultures were fermented at 37 °C and 280 rpm. ClearColi™ cell culture was harvested at 5.4 h (OD_{600} = 1.0), 7.7 h (OD_{600} = 3), and 9.8 h (OD_{600} = 7.0) of fermentation. Cells were homogenized as described above. As with the lyophilized extract, AIM prepared ClearColi™ extract was tested by measuring production levels of sfGFP protein. Similarly, to demonstrate the capacity of this system to produce protein therapeutics, the FDA approved therapeutic crisantaspase was produced.

Results

This project advanced on-demand CFPS systems capable of producing yields appropriate for therapeutic doses. We have achieved an improved lyophilized, endotoxin-free CFPS system from those previously reported by our lab and others. For on-demand CFPS protein production, this new system only requires rehydration with water and the addition of the DNA template of choice. Additionally, the preparation of the endotoxin-free cell extract was optimized for improved preparation efficiency and extract efficacy.

The lyophilized, on-demand CFPS system held activity after storage at elevated temperatures. Samples containing cryoprotectants showed prolonged activity over that of the control system as presented in Figure 3A. The potential of using these lyophilized CFPS systems for protein therapeutic production was demonstrated by the production of the FDA-approved cancer therapeutic crisantaspase. Active crisantaspase was produced at significant yields after 46 days of storage at elevated temperatures (Figure 3B). The promising results of our novel lyophilized on-demand CFPS system demonstrated a viable shelf-stable, one-pot CFPS system utilizing endotoxin-free ClearColi™ cell extract.

The promising results from this study prompted further research into optimizing the preparation of the ClearColi™ cell extract. We demonstrated a streamlined procedure for preparing ClearColi™ cell extract using AIM. This new protocol still requires a greater amount of time than that of traditional BL21 Star (DE3) as shown by the growth kinetics in Figure 4A; however, it significantly reduces required hands-on time. In

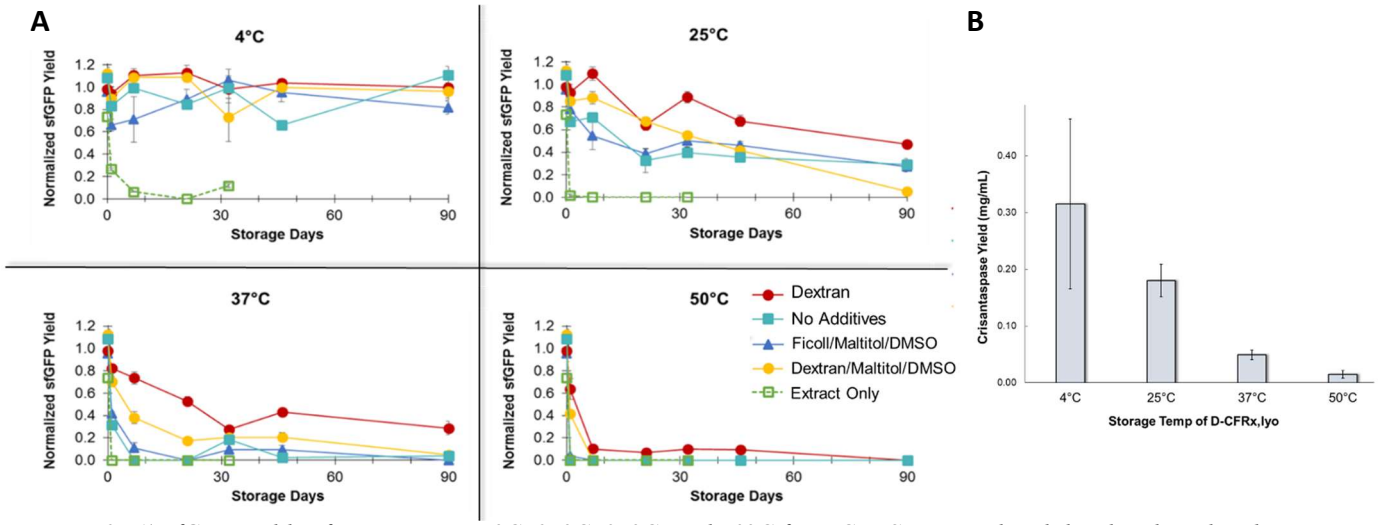

Figure 3: A) sfGFP yields after storage at 4°C, 25°C, 37°C, and 50°C from CFPS systems lyophilized with and without cryoprotectants. Sample combinations are described in Table 1. Extract only sample was not lyophilized with PANOxSP; B) Crisantaspase yields from CFPS system lyophilized with Dextran cryoprotectant after 46 days of storage.

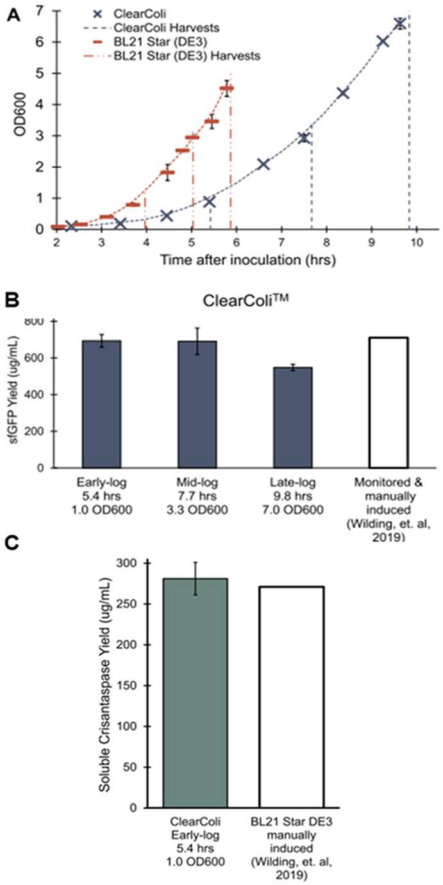

Figure 4: A) Kinetic growth curves (OD_{600}) and harvest times of BL21 Star™(DE3) and ClearColi™ cells prepared in AIM; B) CFPS sfGFP expression yield from ClearColi™ cell extract prepared using AIM as compared to sfGFP yield from ClearColi™ cell extract prepared using traditional fermentation and induction method; C) Crisantaspase yields using ClearColi™ cell extract prepared with AIM compared to yields using traditional fermentation and induction methods.

particular, AIM reduces the need to monitor the cells throughout growth and eliminates manual induction. We report that the optimal time to harvest ClearColi™ to obtain cell extract for high-yielding CFPS is at early to mid-log phase which equates to ~5.5-8 h after initial cell inoculation. Even waiting 10 h after initial cell inoculation still resulted in decent protein expression yields in CFPS (See Figure 4B for protein expression yields at different harvest times). These results suggest that not only does AIM simplify preparation procedures but also allows for more flexibility in the timing of harvest.

The performance of ClearColi™ cell extracts prepared by AIM were further tested by producing the protein therapeutic crisantaspase. AIM prepared ClearColi™ proved to maintain the same cell-free protein synthesis capability as extract produced with traditional fermentation and induction methods as demonstrated by the high-yield expression of crisantaspase as shown Figure 4C. These results suggest that preparing ClearColi™ cell extract with AIM is superior to that of traditional preparation methods as it is significantly more efficient while still maintaining equivalent-yielding CFPS.

A full disclosure and extended discussion of the experimental methods and results of this project are documented in the articles: 1) "Thermostable lyoprotectant-enhanced cell-free protein synthesis for on-demand endotoxin-free therapeutic production" (New BioTechnology), and 2) "Streamlining the preparation of 'endotoxin-free' ClearColi cell extract with autoinduction media for cell-free protein synthesis of the therapeutic protein crisantaspase" (Synthetic and Systems Biotechnology).

Conclusion

Lyophilization, of cell-free systems creates rapid protein synthesis systems that can be created in mass and stored for later use on an on-demand basis. This ready-made powdered CFPS provides high density and non-standard temperature storage improving the overall cost, transportability, and convenience of a CFPS system. Additionally, this unique CFPS system utilizes an endotoxin-free cell extract which eliminates costly and time-consuming purification steps.

This work constitutes an important study for the design of lyophilized CFPS systems for prolonged storage by demonstrating the robust nature and therapeutic production capacity of an on-demand protein production platform. Significant progress has been made towards the development of practical on-demand CFPS

systems for therapeutic protein production during long-term space flight. The advancements made through this project are particularly important in streamlining the production of protein therapetics outside of commercial centralized facilities. In particular, we have simplified the fermentation process of the cell extract, improved CFPS long-term storage, and eliminated endotoxin removal steps. Figure 4 summarizes these advancements.

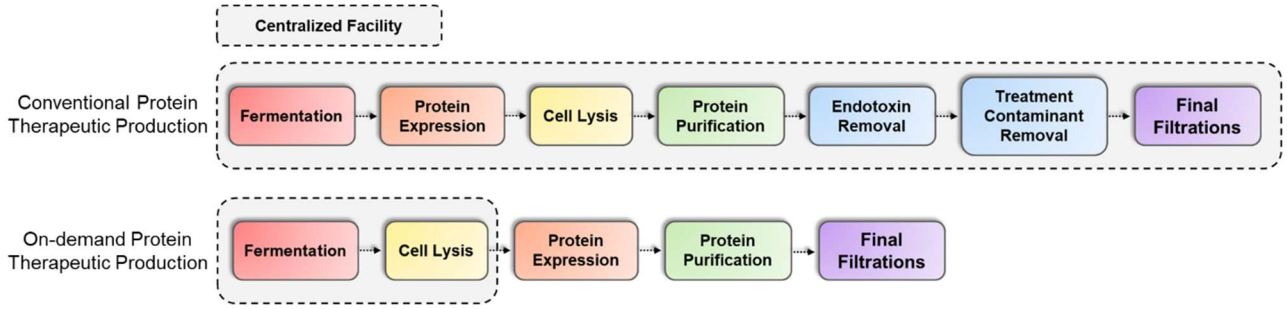

Figure 4: Steps associated with protein therapeutic production through commercial manufacturing (top) and the novel CFPS on-demand , endotoxin-free system described in this work (bottom).

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